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13. ABSTRACT *(Maximum 200 words)*

Lipid-associated membrane proteins (LAMPs) exposed externally on the surface of mycoplasmas are responsible for inducing antibody responses during infections. We showed mycoplasmal LAMPs are species-specific. Antibodies to *M. genitalium* LAMPs detected by ELISA can be confirmed by Western Blotting and consistent with PCR results of patients' urines. We tested more than 1400 serum samples from patients with various diseases. More than 40% of 331 patients attending STD clinics, as opposed to 5-6% of general population, tested positive for *M. genitalium*-specific antibodies. Our results showed there is a hidden epidemic of sexually transmitted *M. genitalium* infection that is apparently clinically silent. We tested 98 serum samples from 43 patients with non-gonococcal urethritis. Among these patients, 63% showed evidence of infections by *M. genitalium*. Infection by *M. genitalium* is statistically significant in association with development of NGU. Our study also revealed the rate of *M. genitalium* infection in women is 4-5 fold higher than that in men. Thus, more women suffer an occult chronic infection by this mycoplasma. In addition to the acute illness associated with the mycoplasmal infection, our study revealed chronic persistent infection by mycoplasmas may also play an important role in human diseases.

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S K M Chung (for) Dec 29, '95

PI - Signature Date

(4) **TABLE OF CONTENTS:**

(5) INTRODUCTION:

Mycoplasma genitalium was originally isolated from the urethra of two homosexual patients with non-gonococcal urethritis (1). Although subsequent studies suggest the organism is present in the urogenital tract of some male and female patients (2), no further isolation of the mycoplasma from the urogenital tract has been reported. Recently, the organism, along with *M. pneumoniae* was identified in throat specimens from patients with respiratory diseases, suggesting that the respiratory tract may be the primary site of infection for *M. genitalium* (3). Interest in this mycoplasma increased following a report from Luc Montagnier and associates detecting the organism in blood samples of one patient with AIDS by PCR assay (4). So far, *M. genitalium* has not been isolated from any clinical samples derived from patients infected with HIV-1 or with AIDS. This is most likely due to the well-known fact that the organism is too fastidious to grow in present culture environments.

More recently, PCR detection of infections with *M. genitalium* is associated with development of non-gonococcal urethritis (NGU) (5). Since it can be extremely difficult to detect mycoplasma(s), many diseases that have been associated with etiologically unknown infections of urogenital system are believed to be caused by mycoplasmas, including pelvic inflammatory diseases (PID). In order to study the actual distribution of *M. genitalium*, the scope of its infection, the mode of its transmission, and the associated disease process of infection by this fastidious mycoplasma, a serological technique capable of detecting the infection with high sensitivity is needed. The test must adequately differentiate *M. genitalium* with high specificity from other mycoplasmas, especially *M. pneumoniae*, which apparently shares many *M. genitalium* antigenic properties. Many individuals may already have significant antibody titers to *M. pneumoniae* due to previous exposure to the agent causing most community-acquired pneumonia.

Mycoplasmal lipid-associated membrane proteins (LAMPs) are exposed on the cell surface (6), are highly antigenic (6,7), and are the most likely immunogenic targets for hosts' responses in mycoplasmal infections. Antibodies to LAMP antigens of each mycoplasma species are highly species-specific and do not cross react with those of other species. We recently developed serological assays to detect specific antibodies to *M. penetrans* using LAMPs (8,9). These assays clearly demonstrated specificity and validity when used on clinical samples from various patients for *M. penetrans, M. salivarium,* and *M. pirum* (8). The successful identification of specific target antigens in mycoplasmas and development of sensitive serological tests to detect their specific antibodies provides scientists and clinicians a powerful tool for epidemiological studies of various human mycoplasmal diseases.

(6) BODY:

1) Experimental Methods:

I. ELISA and Western blotting serological assays: *M. genitalium* and *M. pneumoniae* were grown in tissue culture flasks with SP4 medium. The technique of extracting mycoplasmal LAMPs by Triton X-114 (TX-114) phase fractionation was previously described in detail by our laboratory (8,9). This same technique, with slight modification of our previous method designed for *M. penetrans*, will be used to prepare *M. genitalium* and *M. pneumoniae* LAMPs. Most importantly, the pH of the TX-114 phase fractionation solutions were adjusted to accommodate a maximum extraction of LAMPs for each different species of mycoplasma. The detergent phase after repeated fractionations will be saved, designated as TX-114 extract, and used as antigens for ELISA and Western blot analyses in this study.

Serum Sample Preparation:

1. Serum or plasma samples should be handled as potential infectious agents with precaution according to the institute's guidelines.
2. Undiluted samples are stored at 5°C for short-term use (within a month), otherwise stored at -70°C.
3. Make a 250-500 µl of 5-fold to 10-fold diluted solution for each serum sample in diluent I. Store these diluted samples at 5°C.

ELISA:

1. Thaw one tube of TX-114 extract at room temperature (25°C). Mix by vortexing and store in ice.
2. Make a 1:100 dilution of TX-114 extract in the ELISA plate coating buffer at 25°C in a 50 mL polypropylene conical tube. The concentration of protein is about 2 µg/mL.
3. Coat the Nunc-Immuno F96 MaxiSorp plate with 100 µl solution in each well using Eppendorf repeater pipettor and sterile combitip. Note: We have found this type of ELISA plate most suitable due to the presence of detergent in the coating solution even at a very low concentration (<0.002%), the other kind of plate (PolySorp) from the same company has been found unworkable.
4. Cover the top of ELISA plate with a sealing tape, place the plate in a plastic box with a sheet of prewetted gel blotting paper on the bottom, and incubate at 37°C for 4 h.
5. Aspirate the coating solution and wash the plate twice with buffer W. Invert the plate and tap on two sheets of absorbent paper to remove residual fluids.

6. Overcoat the plate by adding 200 µl of 0.1% BSA, 0.02% sodium azide to each well and incubate at room temperature (25°C) for 2 h.
7. Repeat step 5. At this stage, the ELISA plate can either be stored at -70°C for as long as 6 months or be processed for the next step. (For storage, seal the top of plate with tape and then wrap in a plastic bag.)
8. Pipette 100 µl of diluent II to each well, and then add 2-10 µl of each 5X diluted serum sample depending on the final tested dilution. The first well on the plate should be set aside for blank without adding serum sample.
9. Rock the plate on a orbit shaker at room temperature for 3 min. Incubate at 5°C overnight, and then at 37°C for 2 h.
10. Wash the plate six times as in step 5.
11. Prepare 1:1,000 diluted biotin-labeled goat anti-human IgG-r (0.5 mg/mL stock in 50% glycerol stored at -20°C) in diluent III. Add 100 µl of this solution to each well, and incubate at 37°C for 90 min.
12. Wash the plate as described above. Add 100 µl of 1:20,000 diluted peroxidase-labeled streptavidin (0.5 mg/mL stock)in diluent III to each well and incubate at 37°C for 90 min. Note: There are great differences in term of enzyme activities for peroxidase-labeled streptavidin from different vendors. It is necessary to titrate each batch of enzyme to find the appropriate dilution.
13. Wash the plate six times as in step 5. Prepare 1:1 (v/v) mixture of hydrogen peroxide solution and ABTS substrate solution. Warm up the mixture at 37°C for 10 min.
14. Add 100 µl of the ABTS-H_2O_2 mixture to each well. Develop the color reaction at 37°C for 20 min.
15. Stop the reaction by adding 100 µl of 1% SDS (ABTS stop solution) to each well.
16. Measure the optical density (OD) of each well at 405 nm corrected with a reference wavelength at 650 nm and substrate the OD of the blank.

Western blotting

Proteins (about 90 µg) from *M. genitalium* TX-114 extract were separated by SDS-polyacrylamide gel electrophoresis and electroblotted on a BA-85 nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 5% fetal bovine serum and 1% BSA in PBS pH 7.2 and cut into 4 mm strips. Each strip was incubated for 5 min. with 1/250 human serum 25°C for 15h with shaking. The strips were washed six times with solution A (PBS, pH 7.2 plus 0.05% Nonidet P-40 [NP-40]), incubated at 25°C with 1/1000 biotin-labeled antibody of goat anti-human IgG-g, incubated at 25°C with 1/10,000 peroxidase-labeled streptavidin in diluent I (10% normal goat serum, 2% BSA, an 0.1% NP-40 in PBS)

for 2 h, and developed at 37°C for 20 min. with the 4-chloro-1-naphthol peroxidase substrate system (KP).

II. PCR assay of *M. genitalium*: Since *M. genitalium* is essentially uncultivable, the best standard of positive identification for infection with this mycoplasma is probably a good PCR test. However, preparation of clinical samples, especially for those suspected to carry PCR inhibitory factor(s), as well as analysis of artifacts or contaminations possibly introduced at any step of the procedures have to be meticulously monitored. The complications have prevented most clinical microbiology laboratories from using PCR as a general tool for diagnosis of mycoplasmal infections. Our laboratory has set the standard for PCR detection of mycoplasmas in clinical specimens (10). But, one really has to know first where the primary site of infection is for a particular mycoplasma, before a meaningful assessment of infection rate or incidence by PCR can be accomplished. In this case, *M. genitalium* is an urogenital mycoplasma and its infection is thought to be associated with development of NGU (5,11). Thus, we think noninvasive urine samples should be the most reasonable specimens to study. Urine samples from 100 patients tested positive for antibodies to *M. genitalium* and 100 patients tested negative will be examined by PCR. The urine samples will be processed for PCR according to our previously described methods (4). The oligonucleotide sequences for detection of *M. genitalium* by PCR will be similar to those as described (11). The sequences are as follows:

RW013: 5'-AGT TGA TGA AAC CTT AAC CCC TTG-3'
RW014: 5'-GCA CCG TTG AGG GGT TTT CCA TTT-3'
RW015: 5'-GAC CAT CAA GGT ATT TCT CAA CAG-3'

Oligonucleotides RW013 and RW014 will be used as primer set in the PCR to generate a 284-bp product. The specificity of the PCR product will be determined by its size upon agarose gel electrophoresis, and further verified by hybridization with ^{32}P-labeled oligonucleotide RW015. The results of both tests will be tabulated and compared for sensitivity and specificity for both assays.

2) Results:

(1) Immune reactivity to *M. genitalium* in normal population: Using ELISA and LAMPs as target antigens, we first examined antibodies reactivity in a healthy normal population. Serum samples from normal blood donors (n=384) who donated their blood at the NIH clinical center blood bank were studied. Less than 3% of samples had an OD 405 nm reading higher than 1.0 (Figure 1).

(2) Immune reactivity to *M. pneumoniae* in the same group of blood donors: Using ELISA and LAMPs from *M. pneumoniae* as target antigens, we measured antibody activity in this healthy control population. We found more than 70% of individuals produced OD reaction equal to or higher than 1.0 (Figure 2). The difference in distribution of reactivity to *M. genitalium* and to *M. pneumoniae* among these individuals clearly demonstrates there is little antigenic cross reactivity for LAMPs from these two closely related species. More specifically, antibodies to *M. pneumoniae* LAMPs apparently will not react with *M. genitalium* LAMPs.

(3) Using the OD readings from normal blood donors (n=384), we determined the cut-off for positive antibody reaction as: mean + (3 x S.D). Patients attending STD clinics and HIV-infected patients with or without clinical AIDS (AD) were found to be highly prevalent for *M. genitalium* infection (Figure 3). Only 2-5% of patients with malignant diseases and healthy control blood donors tested positive for *M. genitalium*-specific antibodies. We also found that 139 out of 331 patients attending STD clinics (42%) tested positive for the antibodies.

(4) Serum samples of 100 patients with AIDS, HIV-positive asymptomatic blood donors (AD) and STD clinic patients that tested positive for *M. genitalium*-specific antibodies in ELISA were then studied by WB. Figure 4 reveals the pattern of positive reactivity to *M. genitalium* LAMPs on WB (Lanes G-N). Sera that have strong positive reactivity to *M. pneumoniae* LAMPs (Lanes D-F) do not react with *M. genitalium* LAMPs. Essentially all serum samples that tested positive with ELISA could be confirmed by WB analysis.

(5) To correlate the antibody test with PCR results, urines were obtained from 68 HIV-positive AD patients and 36 intravenous drug users (IVDUs). PCR results for *M. genitalium* DNA in urines from these patients were tabulated with results of their serological tests for *M. genitalium*-specific antibodies (Table 1). It is interesting to note that all the patients testing positive for *M. genitalium* in urines by PCR also tested positive for antibodies. On the other hand, more than half of the patients who were positive for the antibodies tested negative by PCR for *M. genitalium* in urines. It is not clear if these patients could have had *M. genitalium* infection(s) at different anatomical sites such as respiratory tract or GI tract. However, serological assay appears to be more sensitive than PCR testing in screening for evidence of *M. genitalium* infection.

(6) The seroepidemiological results were further analyzed to evaluate difference in frequencies of *M. genitalium* infections between women and men in the general population as well as in STD clinic patients. All samples from patients with known gender were tabulated (Table 2). Women

appeared to have significantly higher incidence of *M. genitalium* infection than men in the normal general population (10.2% versus 2.5%; odds ratio [OR] = 4.4; $\chi^2 = 9.7$; $p < 0.01$). The incidences of *M. genitalium* infection highly increased in both women and men patients attending STD clinics. For women, the incidence increased from 10.2% to 40.7% (OR = 6.0; $\chi^2 = 35.4$; $p < 0.0001$). For men, the incidence increased from 2.5% to 34.1% (OR = 19.9; $\chi^2 = 64.2$; $p < 0.0001$). Interestingly, difference in the incidences of *M. genitalium* infection between women and men patients attending STD clinics was not significant (40.7% versus 34.1%; OR = 1.3; $\chi^2 = 1.1$; $0.20 < p < 0.30$).

(7) Like many viral infections in human urogenital tracts, mycoplasmal infections in the urogenital tracts are often clinically silent. However, it has long been suspected that many cases of patients with NGU may be due to infection of fastidious mycoplasma(s) such as *M. genitalium.* In order to study the possible role of *M. genitalium* infection and development of NGU, we tested 98 serum samples from 43 patients with clinical complaints of NGU. We found 63% of these patients (27 out of 43) tested positive for *M. genitalium.* In this study, only 23 out of 384 healthy blood donors (6%) tested positive for the *M. genitalium*-specific antibodies. Thus, compared to those showing no evidence of *M. genitalium* infection, the relative odds of developing NGU for the patients infected by *M. genitalium* was 26.5 (OR = [27 x 361] / [16 x 23] = 26.5; $\chi^2 = 120.7$; $p < 0.0001$). Unfortunately in this study, we do not have clinical information about the presence or absence of NGU symptoms for our STD clinic patients who showed high frequency of *M. genitalium* infection. However, we assume, even though it is unlikely, that none of them developed NGU. Analyzed against the high background of these STD clinics' patients, the relative odds of developing NGU for patients infected by *M. genitalium* was still statistically significant (OR = [27 x 192] / [16 x 139] = 2.33; $\chi^2 = 6.67$; $p < 0.01$).

3) Discussion:

In less than 1 year, we have accomplished many goals listed in our original research proposal. We also have several important findings pertinent to women's health and general biomedical information.

(1) **To examine if LAMPs of *M. genitalium* are truly species-specific in human serological immune responses.** We demonstrate there is little cross reactivities in human serological immune response to LAMPs of *M. genitalium* or LAMPs of *M. pneumoniae.* More than 80% of individuals in the general population are positive for antibodies to *M. pneumoniae.* But, only 5-6% have positive antibodies to *M. genitalium.*

(2) **To confirm positive ELISA seroreactivity by Western Blot analysis in patients attending STD clinics.** All sera that tested positive to *M. genitalium* LAMPs in ELISA can be confirmed by Western blotting. Thus, the antibodies produced in human immune response to infections by *M. genitalium*, are reacting to the protein moiety of mycoplasmal lipid-modified surface proteins.

(3) **To evaluate the validity and sensitivity of the mycoplasmal LAMPs antibody tests by PCR study of *M. genitalium* in urines from at least 50 patients.** We studied urine samples from 104 patients by PCR to detect *M. genitalium.* All the patients that tested positive for *M. genitalium* by PCR had positive antibodies to the LAMPs of the mycoplasma. The LAMPs antibody test is much more sensitive than the PCR assay for *M. genitalium* DNA.

(4) **To examine incidence of *M. genitalium* infection in different groups of patients by the serological test.** Using ELISA for antibodies to *M. genitalium* LAMPs, we have examined more than 1400 serum samples from normal blood donors, patients with AIDS, HIV-infected asymptomatic patients (AD), STD clinics' patients, patients with malignant diseases and patients with clinical symptoms of NGU. It appears that HIV-infected patients with or without clinical AIDS as well as HIV-negative patients attending STD clinics have high frequencies of *M. genitalium* infection.

(5) **Women appear to have significantly higher incidence of infection by this urogenital mycoplasma.** The difference in incidences of *M. genitalium* infection between men and women is particularly significant in the general population. The organism apparently can colonize in women's urogenital tracts more easily. The frequency of *M. genitalium* infection in both men and women attending STD clinics markedly increased. Thus, there is a hidden epidemic of sexually transmitted mycoplasmal infection due to *M. genitalium.* Among STD clinics' patients, women still have higher rate of *M. genitalium* infection than men. However, the difference between men and women becomes less marked.

(6) **Patients with evidence of *M. genitalium* infection is statistically significant in association with development of NGU.** In our case-control study, the relative odds of developing NGU for the patients who test positive for *M. genitalium* antibodies, compared to those who showed no sero-evidence of *M. genitalium* infection, is 26.5 times greater. Thus, although most of mycoplasmal infections are clinically silent, infection by *M. genitalium* in patients is significantly associated with development of clinical symptoms of NGU.

(7) CONCLUSIONS:

We have developed a highly specific and sensitive serological assay for antibodies to *M. genitalium*. Mycoplasmal LAMPs are species specific. There is little antigenic cross reaction between the two closely related species of human mycoplasma, *M. genitalium* and *M. pneumoniae*. The validity of the antibody test can also be demonstrated by independent PCR study. Using this powerful serological test, we found that *M. genitalium* is indeed a previously unrecognized sexually transmitted urogenital mycoplasma. More than 40% of patients attending STD clinics test positive for *M. genitalium*-specific antibodies copared to only 5-6% of the general population. More importantly, our preliminary results indicate the rate of *M. genitalium* infection in women is 4-5 fold higher than that in men. Because there was no test available previously, infections by *M. genitalium* have not been recognized. Our study shows there is an epidemic of sexually transmitted *M. genitalium* infection. We also demonstrate that patients infected by *M. genitalium* have a very high possibility of developing NGU. The result strongly suggests that a significant percent of NGU may be due to *M. genitalium* infection. Like many viral infections, most mycoplasmal infections are also clinically silent. Evidently, more women suffer an occult chronic infection by this mycoplasma without acute clinical symptoms and may be the reservoir for this mycoplasma. In future studies, it will be important to study physiological factors in women that favor mycoplasmal colonization. We believe chronic persistent infection by the mycoplasma, even without an acute illness, may have an important clinical impact in development of chronic debilitating diseases. Our laboratory recently reported that chronic persistent infection of mycoplasmas may transform normal mammalian cells into cancerous malignant cells (12). Different from viral infections, mycoplasmal infections are treatable and may even be curable once the infections are diagnosed. Our findings should have a very significant implication in clinical care of patients, especially for women who are apparently more likely to be colonized by various species of mycoplasma. The results of this study are in preparation for publication. In our continuing study of patients attending STD clinics, we will also specifically examine various urogenital diseases and cytopathological changes in women who have seroevidence of *M. genitalium* infection.

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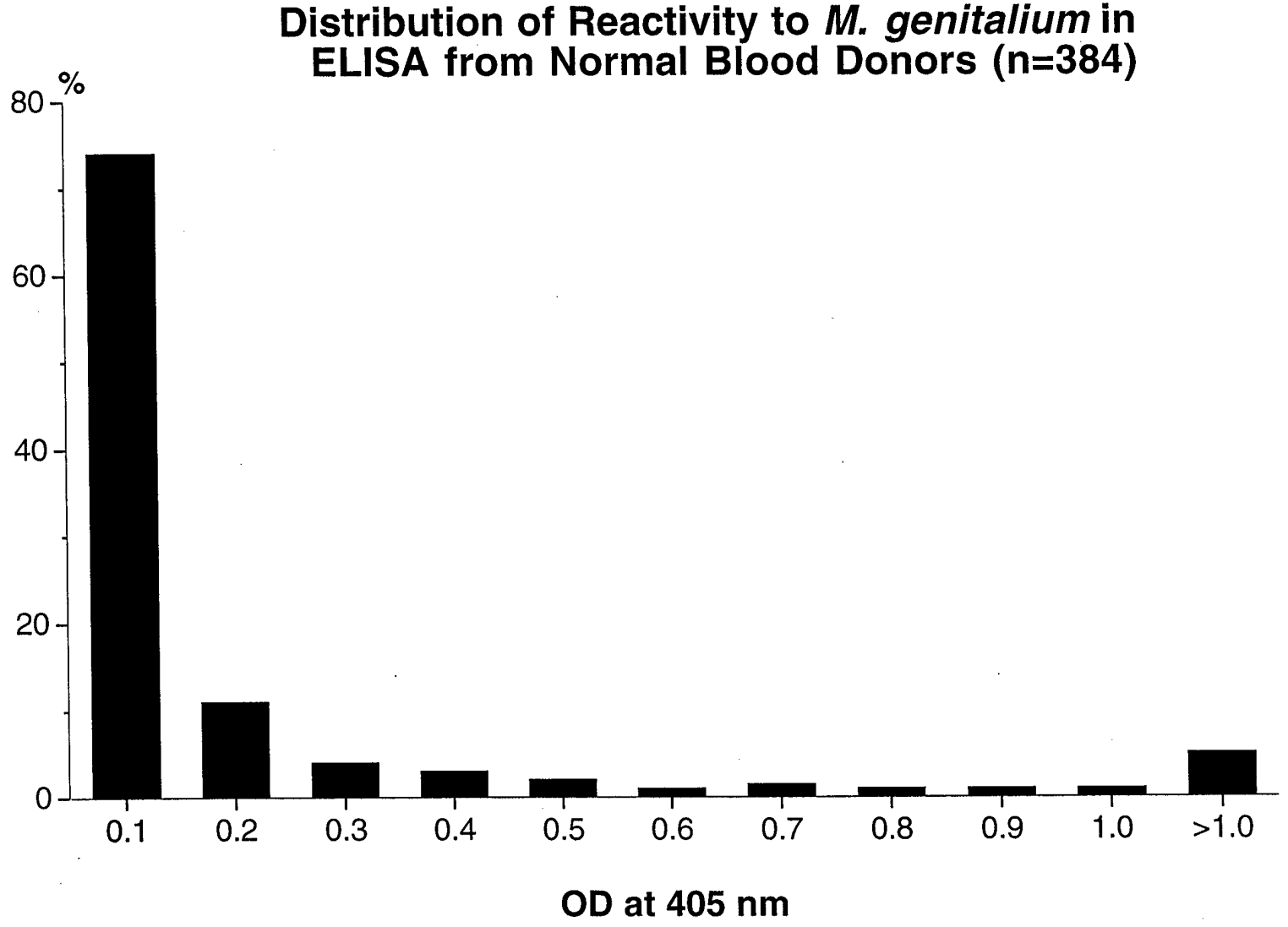
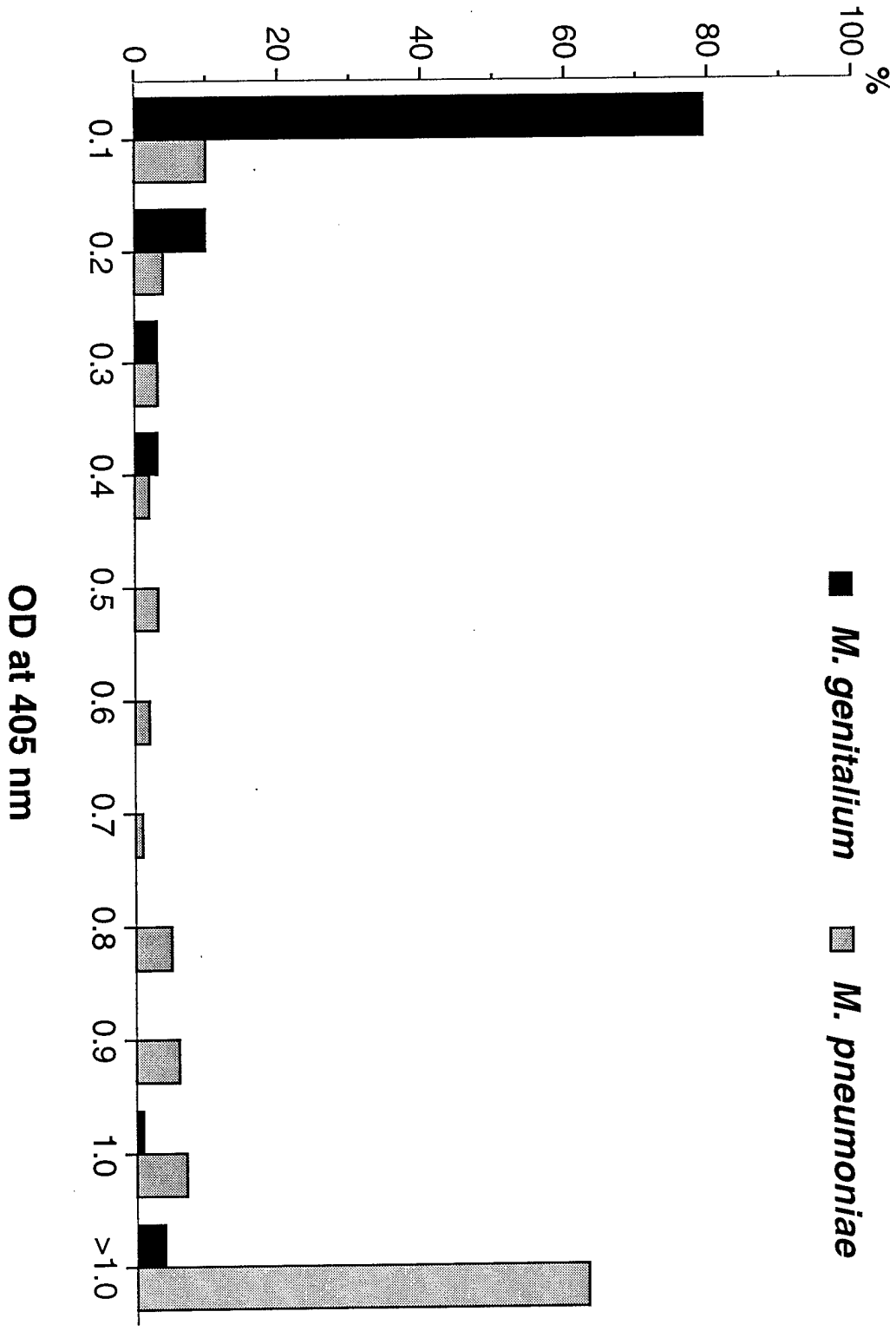

Distribution of Reactivity to M. genitalium in ELISA from Normal Blood Donors (n=384)
%
80
60
40
20
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
>1.0
OD at 405 nm

FIGURE 1

FIGURE 2


0
20
40
60
80
100
%
0.1
0.2
0.3
0.4
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0.6
0.7
0.8
0.9
1.0
>1.0
OD at 405 nm
M. genitalium
M. pneumoniae

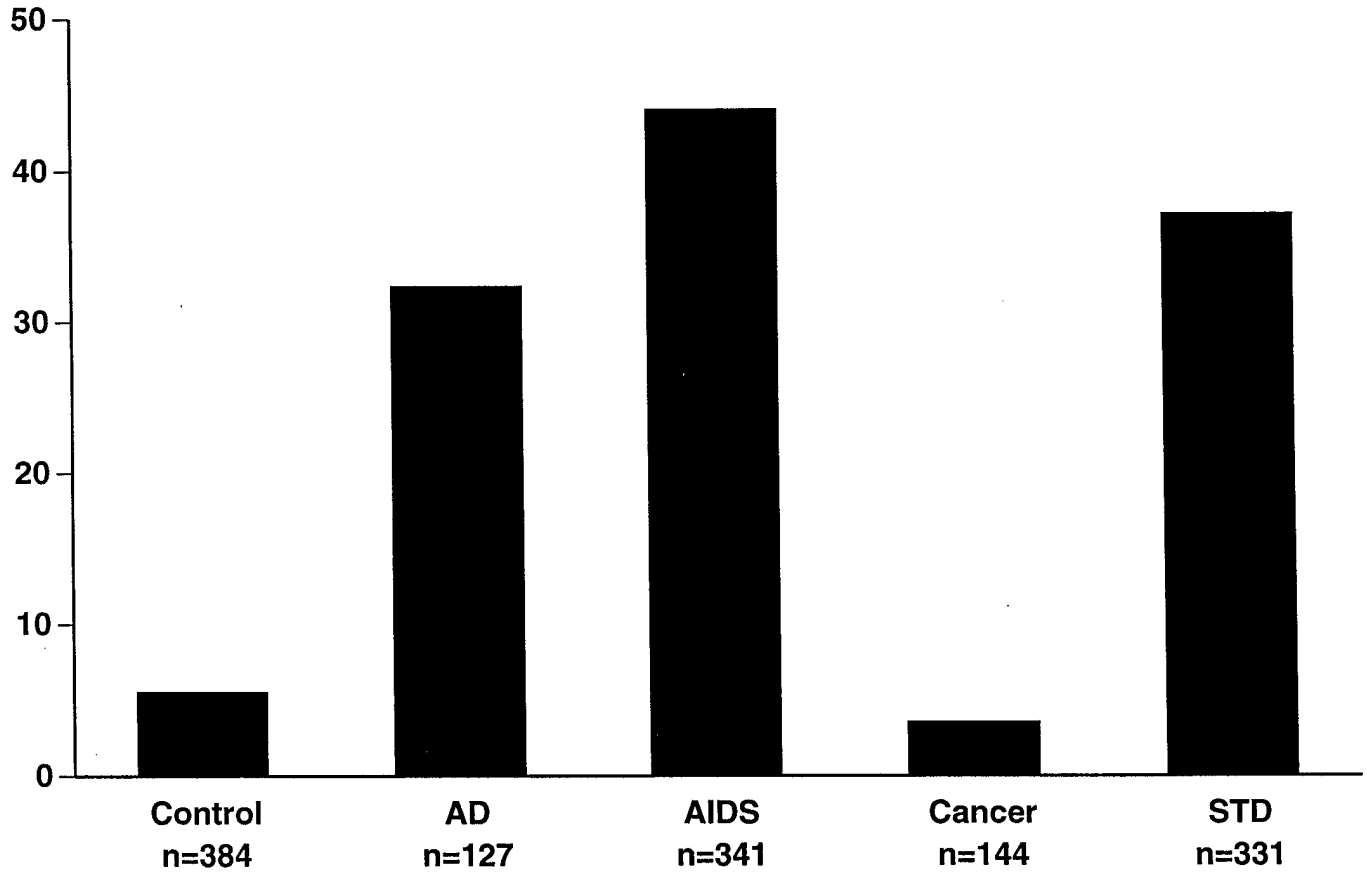
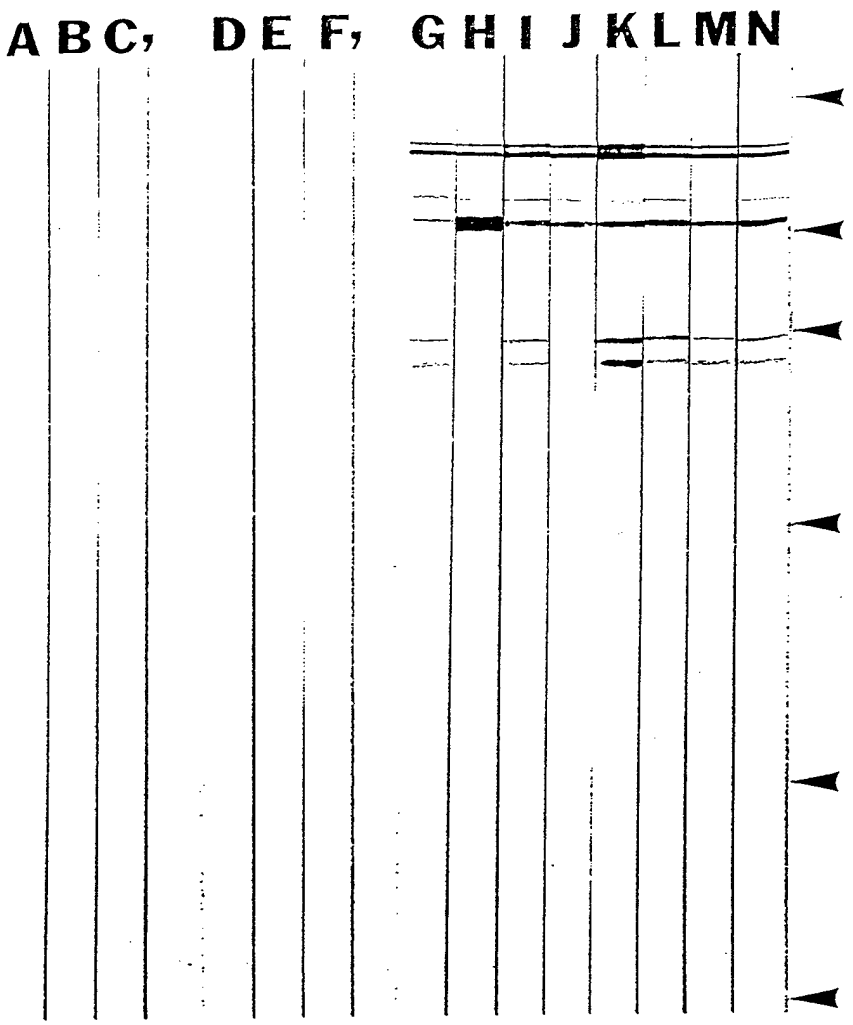

Antibody Positivity to *M. genitalium* LAMP Antigens
50
40
30
20
10
0
Control
n=384
AD
n=127
AIDS
n=341
Cancer
n=144
STD
n=331

FIGURE 3

FIGURE 4



Western blot anaylsis of antibody reactivity to *M. genitalium* LAMP antigens. Lanes A-C, sera from 3 HIV-negative healthy blood donors. Lanes D-F, sera from 3 HIV-negative individuals having positive reactivity to *M. pneumonia* but negative reactivity to *M. genitalium* in ELISA. Lanes G-N, sera from HIV-infected patients (G to K) and HIV-negative patients attending STD clinics (L to N) having positive reactivity to *M. genitalium* in ELISA. Arrows indicated the position of prestained protein size markers with apparent molcular weight from top to bottom of 205kd (kilodalton), 103kd, 67kd, 42kd, 28kd, and 18kd, respectively.

Gender Differences in Incidence of *M. genitalium* Infection

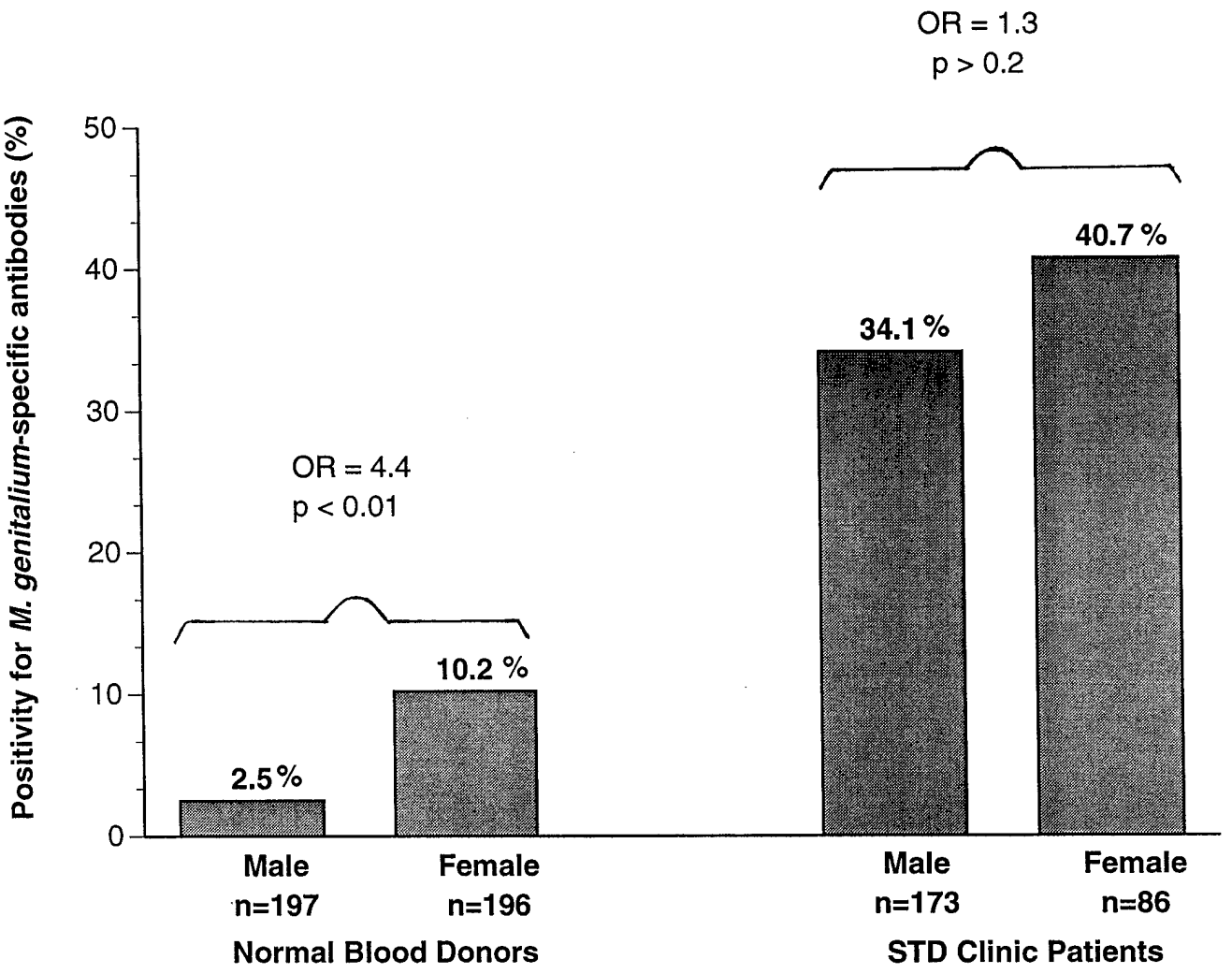


FIGURE 5

FIGURE 6

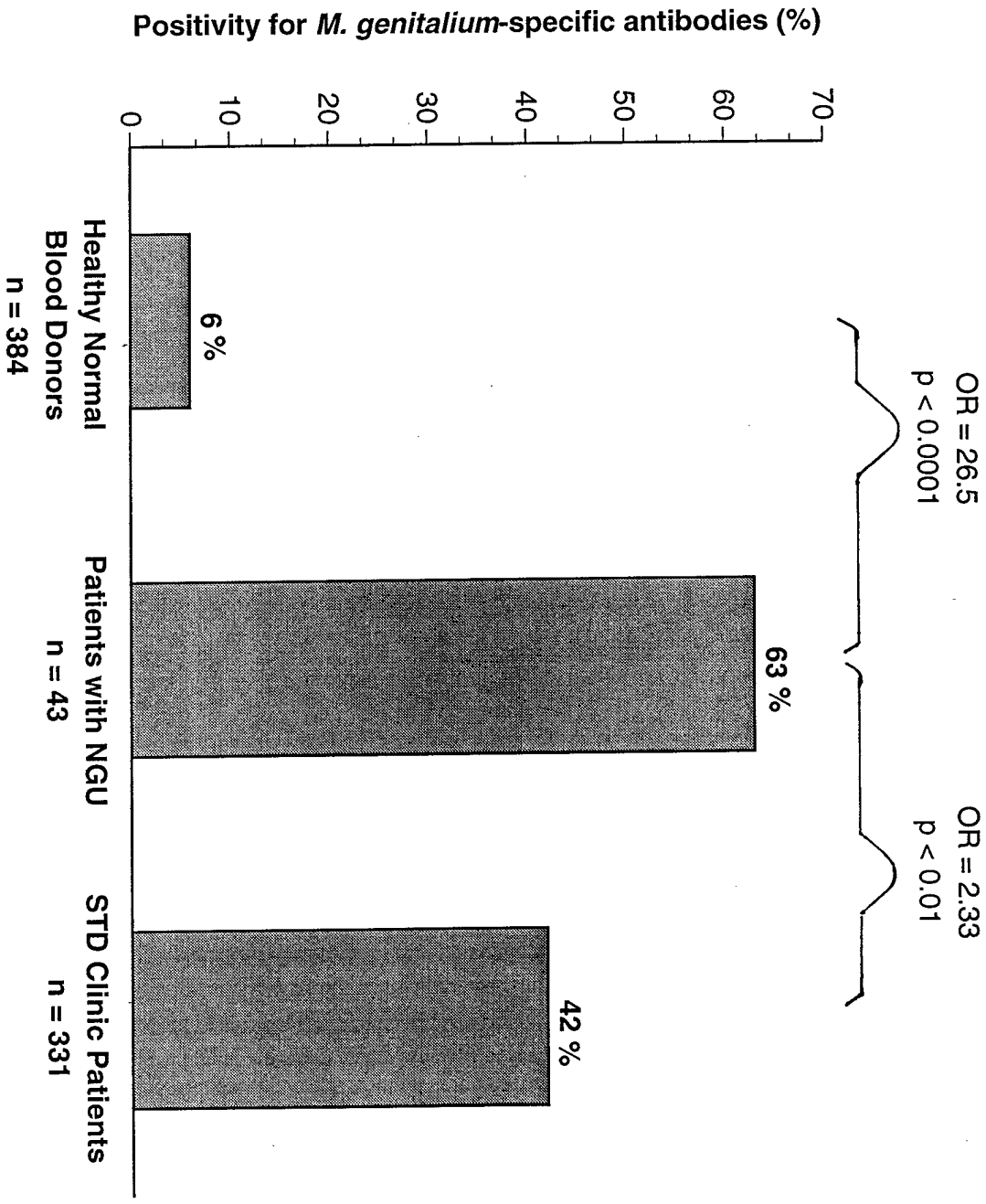

Positivity for *M. genitalium*-specific antibodies (%)
0
10
20
30
40
50
60
70
6 %
63 %
42 %
Healthy Normal Blood Donors n = 384
Patients with NGU n = 43
STD Clinic Patients n = 331
OR = 26.5 p < 0.0001
OR = 2.33 p < 0.01

TABLE 1

M. genitalium Infection Detected by Positive Serum Antibodies to LAMPs and by PCR of Urine Samples

Patient group	Number Tested		ELISA	Urine PCR
HIV$^+$-AD	68	+	24	7/24
		–	44	0/44
IVDU	36	+	16	8/16
		–	20	0/20